



HAL
open science

Kawal: A fermented food as a source of Bacillus strain producing antimicrobial peptides

Blaise Waongo, Maude Pupin, Matthieu Duban, Gabrielle Chataigne,
Oumarou Zongo, Hama Cisse, Aly Savadogo, Valérie Leclere

► To cite this version:

Blaise Waongo, Maude Pupin, Matthieu Duban, Gabrielle Chataigne, Oumarou Zongo, et al.. Kawal: A fermented food as a source of Bacillus strain producing antimicrobial peptides. *Scientific African*, 2023, 20, pp.e01714. 10.1016/j.sciaf.2023.e01714 . hal-04426241

HAL Id: hal-04426241

<https://hal.science/hal-04426241>

Submitted on 5 Apr 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Kawal: A fermented food as a source of *Bacillus* strain producing antimicrobial peptides

Blaise Waongo^{a,b,c}, Maude Pupin^b, Matthieu Duban^c, Gabrielle Chataigne^c, Oumarou Zongo^a, Hama Cisse^a, Aly Savadogo^{a,*}, Valérie Leclere^c

^aLaboratory of Applied Biochemistry and Immunology, University Joseph KI-ZERBO, Ouagadougou 03, BP 7021, Burkina Faso

^bUniv. Lille, CNRS, Centrale Lille, UMR 9189 CRISTAL, Lille F-59000, France

^cUniversity of Lille, Secondary Metabolites of Microbial Origin, Charles Viollette Institut, UMRT 1158 BioEcoAgro, Lille F-59000, France

ARTICLE INFO

Article history:

Received 9 January 2023

Revised 23 April 2023

Accepted 12 May 2023

Editor: DR B Gyampoh

Keywords:

Kawal

Bacillus

NRPS

Antimicrobial peptides

ABSTRACT

Kawal is a fermented food condiment obtained by spontaneous fermentation of *Senna obtusifolia* leaves. Fermentative microbiota consists of microorganisms producing a diversity of bioactive molecules. Thus, research of novel microbial products capable of inhibiting or eliminating plant and food pathogens is an absolute necessity. In this study, 126 bacterial strains were isolated from *Kawal*. Phenotypical, biochemical and genotypic methods were used to determine their genus or species. Forty-three (43) of them belonging to the genus *Bacillus* were selected for their antibacterial and antifungal properties. Their ability to produce lipopeptides (fengycins, surfactins, iturins, kurstakins) was investigated using HPLC and Matrix-assisted Laser Desorption Ionization-Time of Flight (MALDI-ToF-MS). Molecular characterization of 16S rRNA identified 10 species belonging to the genus *Bacillus*: *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. velezensis*, *B. spizizenii*, *B. wiedmannii*, *B. tropicus*, *B. cereus*, *B. tequilensis* and *B. safensis*. MALDI-ToF-MS analysis revealed a diversity of antimicrobial molecules. Among all *Bacillus* strains tested, 84.44% produced surfactin, 22.22% produced fengycin, 11.11% produced iturin and 2.22% produced kurstakin. The results of this study demonstrated the high diversity of lipopeptides among *kawal Bacillus* strains and their antimicrobial capacity. In view of the potentialities that have been highlighted in these strains, some of them can be used in the bioconservation or in the bioprotection of plants.

© 2023 The Author(s). Published by Elsevier B.V. on behalf of African Institute of Mathematical Sciences / Next Einstein Initiative.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

Introduction

Kawal is a fermented food condiment obtained by natural and alkaline fermentation of the leaves of *Senna obtusifolia* also called *Cassia obtusifolia*. This plant is known by several vernacular names in Burkina Faso: Katre-nangouri in Moore, Krikri in Dioula, Jangboro in Lobi, kouéré in Bwamu and youlou-sonan in Gourunssi [1]. *Kawal* is very appreciated and widely

Abbreviations: NRPS, Non Ribosomal Peptide Synthetases; PKS/NRPS, Polyketide Synthetases/Non Ribosomal Peptide Synthetases; LB, Lysogeny Broth; ESI, Electro-Spray Interface; PCR, Polymerase Chain Reaction; M. luteus, Micrococcus luteus; B., Bacillus.

* Corresponding author.

E-mail address: alysavadogo@gmail.com (A. Savadogo).

<https://doi.org/10.1016/j.sciaf.2023.e01714>

2468-2276/© 2023 The Author(s). Published by Elsevier B.V. on behalf of African Institute of Mathematical Sciences / Next Einstein Initiative. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

consumed by the African populations. Traditional techniques and processes for making *kawal* are pounding, fermentation and drying. Indeed, the leaves are harvested at the end of the rainy season when the plant is well developed. Then they are cleaned of all foreign matter and impurities such as leaves from other plants, worms, insects, pods and flowers from the plant itself. The selected unwashed leaves are crushed by pounding with a wooden mortar and pestle into a paste. The resulting paste is usually placed in an earthenware jar and buried in a shady place for fermentation, which usually lasts fifteen days [1].

In Africa, the lack of a reliable and sufficient cold chain is one of the main causes of losses of perishable products, estimated at about 25–30 percent for animal products and 40–50 percent for roots, tubers and fruits and vegetables [2]. The fermentation of food products remains a traditional technique easy to implement, allowing the transformation and the conservation of products [3]. Thus, a controlled fermentation of food products requires a mastery of microbiology and the focus of starters [4]. A well selected starter allows to produce under controlled conditions, healthy food with all the organoleptic characteristics of the traditional fermented product [3]. Development and valorization of *kawal*, by biotechnology represent an interesting possibility from sanitary and economic point of view. Abundance of protein, carbohydrates and minerals in *kawal* makes it a food of choice sometimes used by families as a substitute for meat and/or fish [1].

Previous studies on the microbiological characterization of *kawal*, have shown that the dominant microflora consists of spore forming germs of the genus *Bacillus* [5]. No study has so far revealed whether these microorganisms are also responsible for the production of antimicrobial substances of lipopeptic type [6] and for the preservation of this food. These lipopeptides are amphiphilic components with high structural biodiversity and functional properties [7]. They are synthesized by Non Ribosomal Peptide Synthetases (NRPS) or hybrid Non Ribosomal Polyketide and Peptide Synthetases (PKS/NRPS) [8]. *Bacillus spp.* are widely studied for their production of five different families lipopeptides named fengycins/plipastatins, surfactins, iturins, kurstakins and locillomycins [9].

Due to their antimicrobial activity, some of these biosurfactant molecules could be used in food biopreservation or in agriculture as biopesticides instead of chemicals [10].

Fengycins are lipodecapeptides with a peptide chain as Glu-(D-Orn)-D-Tyr-(D-a-Thr)-Glu-(D-Ala/Val)-Pro-Gln-(Tyr)-Ile partially cyclized and linked to a β -hydroxy fatty acid of 14–18 carbon atoms [11]. This family includes fengycin A and B, or plipastatin, if Tyr9 is of D configuration. Surfactins are lipoheptapeptides with different peptide chains linked to a β -hydroxy fatty acid of 13–18 carbon atoms [12]. This family includes variants of esperin, lichenysin, pumilacidin and surfactin. Iturins are lipoheptapeptides with different peptide chains linked to a β -amino fatty acid chain of 14–17 carbon atoms [13]. The main variants of this are: iturin A and C, bacillomycin D, F and L, mycosubtilin and mojavensin. Kurstakins are lipoheptapeptides containing the cyclic or linear peptide sequence (D-Thr)-Gly-D-Ala-Ser-His-(D-Gln)-Gln linked to a fatty acid chain of 11–14 carbon atoms, β -hydroxylated or not [14]. Locillomycins are partially cyclized nonapeptides with the peptide sequence Thr-D-Gln-Asp-Gly-Asn-Asp-Gly-Tyr-Val linked to a fatty acid chain of 13–15 carbon atoms [9].

Among the beneficial traits, the secondary metabolites produced by *Bacillus spp.* are widely applicable in medicine and agriculture for their biological activities [15]. For example, bacteriocins, lantipeptides and polyketides are antibiotics from the genus *Bacillus* that have broad-spectrum antibacterial activities [16]. In the food industry, surfactants are used as texture agents during product processing, for the solubilization of flavors and emulsification. Moreover, the broad antifungal spectrum and the low in vivo toxicity of fengycin or plipastatin allow, for example, to consider their use for the protection of seedlings and crops [11].

Previous studies have demonstrated that surfactin and iturin A have antibacterial activity in suppressing the growth of *Rhizoctonia solani*, *Podosphaera fusca*, and post-harvest fungal pathogens of citrus [16]. Therefore, the aim of this work was to characterize the spore-forming microflora involved in the alkaline fermentation of *Senna obtusifolia* leaves. This constitutes an initial step in the selection process of multifunctional starter cultures for a controlled production of *kawal*.

Material and methods

Isolation, purification and characterization of microorganisms

Twenty-eight samples of *kawal* were purchased in the markets of Abéché, Bokoro, Mandelia and N'Djamena in Tchad and the bacteria were isolated in the Laboratory of Applied Biochemistry and Immunology, Universty Joseph KI-ZERBO.

The isolation of bacteria was performed according to the method described by Savadogo *et al.* [17]. Ten (10) g of the *kawal* were placed in 90 mL of Salted Trypton (Merck, Darmstadt, Germany) sterile water and the mixture was then heated in a water bath at 90°C for 10 min in order to select the spore-forming bacteria. Successive decimal dilutions were made in tryptone salt (Merck, Darmstadt, Germany) water from the heat-treated mixtures. After ten-fold serial dilutions, 0.1 mL of each dilution was spread on the surface of Lysogeny Broth (LB) (Merck, Darmstadt, Germany) agar and incubated at 37°C for 48 h.

The purification of *Bacillus sp.* strains was performed by successive subculturing on LB agar, in order to obtain identical colonies, with the same appearance, shape, size and color. Each colony obtained on nutrient agar was sterilely subcultured in 1.5 mL of heart-brain broth supplemented with 15% glycerol in cryotubes and chilled at +4°C. All the pure culture isolates obtained by repeated streaking were characterized by usual microbiological methods: cell morphology, motility, Gram staining, catalase test, oxidase reaction and spores forming.

Antibacterial activity

Bacillus isolates were used for evaluation of antibacterial activity. The agar well diffusion method described by Tagg *et al.* [18] was used. Each *Bacillus* strain was grown in 10 mL of LB broth at 37°C. After 24 h, the culture was centrifuged at 7000 g for 10 min. The crude supernatant was collected then sterilized by filtration with a 0.22 μm millipore filter.

The antibacterial activity of *Bacillus* strains was tested against *Micrococcus luteus* LMG3293 [19]. A culture of *Micrococcus luteus* (10^6 CFU/mL) was inoculated by flooding on to Petri dishes containing LB agar. Then 6 mm diameter wells were made on the agar and each well is filled with 100 μL of filtered supernatant. The plates were left at 4°C for 1 h to allow diffusion of the supernatant and incubated at 37°C. After 24 h, the antibacterial activity of tested strain was revealed by the presence of a clear zone around the colony.

Antifungal activities

Bacillus isolates that showed significant antibacterial activity were selected for evaluation of antifungal activity according to the methodologies described by Tapi *et al.* [20] and Savadogo *et al.* [17]. Two techniques were used (spot and well) to determine antifungal activities against *Aspergillus flavus* UBOCC-A-106,031, *Aspergillus parasiticus var. globasus* UBOCC-A-111,042 and *Aspergillus fumigatus* S1 isolated and identified from previous work in Burkina Faso [21].

Hemolytic activity

The hemolysis, or lysis of red blood cells, is due to the rupture of their plasma membrane, often under the action of phosphatidyl-choline esterase of bacteria and under the action of molecules causing the formation of membrane spores (Hemolysin). This phenomenon releases hemoglobin which is then more or less digested. The hemolytic capacity of the strains was evaluated according to the methodology described by Savadogo *et al.* [17]. In 100 mL of LB agar medium, 5 mL of horse blood is added. These dishes were inoculated with 100 μL of strain culture and then incubated at the optimal growth temperature (37°C) of the strain for 24 h. The zones of inhibition or halos were observed.

Strain identification

All isolates, which produced antimicrobial compounds of peptidic nature, were identified using 16S ribosomal RNA (rRNA) gene sequencing. The amplification of the 16S rRNA gene was performed using the following primers: forward primer 5'-AGAGTTTGATCMTGGCTCAT-3' and reverse primer 5'-GGMTACCTTGTTACGAYTTC-3'. The PCR mixture was composed of 5 μL of genomic DNA (40–50 $\text{ng}\cdot\mu\text{L}^{-1}$), 2.5 μL of each primer (10 μM), 15 μL of milliQ water and 25 μL DreamTaq PCR Master Mix (2x) from Thermo Fisher Scientific (Waltham, MA, USA). The reaction mixture was incubated in DNA Thermal Cycler (ProFlex™ 3 \times 32-well PCR System, Applied Biosystems, Carlsbad, CA, USA). Cycling conditions were denaturation at 95°C for 10 min, 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min 20 s and one cycle of elongation for 7 min at 72°C. PCR products (5 μL of each PCR content) were electrophoretically separated on a 0.8% agarose gel, in TAE buffer 1 \times (40 mM Tris, 20 mM acetate and 1 mM EDTA) with GelRed® (1:10,000 ratio), at 100 V for 1 h and visualized by UV transillumination using Gel Doc (Bio-Rad, Hercules, CA, USA). PCR products were then purified using the Nucleospin gel and PCR clean up Kit (Macherey-Nagel) and sequenced. Amplified 16S rRNA fragments were sequenced by Eurofins genomics (Nantes, France).

The obtained sequences were compared with 16S ribosomal RNA sequences (bacteria and archaea) and Genbank databases, respectively, using the Nucleotide Basic Local Alignment Tool (BLASTn) software provided online by the National Center of Biotechnology and Information (NCBI, Bethesda, MD, U.S.A.). Sequence alignment and phylogenetic tree construction were performed on the basis of 16S rRNA using MEGA 11 [22].

Detection of the biosynthetic potential of lipopeptides

Research and quantification of lipopeptides by MALDI-ToF-MS

MALDI-ToF-MS was used to screen lipopeptides from whole bacterial cells on solid media. Cultures were performed on LB agar plates according to Vater *et al.* [23] and incubated at 37°C for 24 hours. Thus, 1 μL of cell material was picked from plate's agar with a pipette tip. The tip with cells was deposited into an eppendorf tube containing 20 μL of a saturated solution. This solution consists of α -cyano-4-hydroxycinnamic acid in a 1:2 v/v solution of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ with 0.1% trifluoroacetic acid. The tubes containing this solution were vortexed for 30 s. A volume of 1 μL was deposited on the target plate and let dry before analysis. Analysis was made on a Bruker Daltonics MALDI-ToF-MS Ultraflex (Bruker, Bremen, Germany) equipped with a nitrogen laser operating at 337 nm, laser power 116.8 μJ , accelerating voltage 20 kV, positive mode, reflectron mode, and matrix suppression up to 500 m/z. The instrument was calibrated with a peptide mix ranging from 1046.54 to 3147.47 (Bruker part.206195). The spectrum was obtained with 5 \times 30 shots on the sample. Analyses were performed on two to four different samples of each type (colonies of one strain).

Lipopeptides quantification by HPLC-MS

The analyses of HPLC coupled to mass spectrometry were performed on an Accela UHPLC system acquired from Fisher Scientific (Thermo Fisher Scientific, Bremen, Germany) that was composed of an auto-sampler equipped with a column oven, a tray compartment cooler and a quaternary pump with a built-in solvent degasser, all piloted by Xcalibur software. The chromatographic separation was performed with the same column used for standard HPLC analysis. Samples elution was performed with a constant flow rate of $600\mu\text{L}\cdot\text{min}^{-1}$ and the eluent was a gradient composed of solvent A (water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid). The gradients for the separation of each lipopeptide used solvents A and B to compose eluents as first described, whereas trifluoroacetic acid is replaced by formic acid for better compatibility with the MS. The Accela UHPLC system was coupled to an Orbitrap mass spectrometry system (Thermo Fisher Scientific, Bremen, Germany). A flow splitter was used as the output rate of HPLC was higher than that tolerated by the mass spectrometer. The flow was split to give approx. $200\mu\text{L}\cdot\text{min}^{-1}$ directed into a mass spectrometer via the electrospray interface (ESI). The system was equipped with an ESI interface that was used in a positive ionization mode with the following conditions: capillary temperature and voltage at 275°C and 9V respectively, ion spray voltage at 3.3 kV and tube lens voltage at 95V . Nitrogen (N_2) was used as the sheath gas and helium (He) as auxiliary gas with a flow rate of 25 and 5 arbitrary units. For MS experiments, full MS scan was applied, for which the spectra were recorded in the range of m/z 100–2000 with a resolution of 30,000. An external calibration of the equipment for mass accuracy was carried out the day before the analysis according to the manufacturer's guidelines.

Statistical analysis

Experiments were performed at least in duplicate. Data were analyzed using Microsoft Excel Office 2016 and R software version 4.2.0.

Results

Phenotypic characteristics of isolates

A total of 126 strains were isolated, including 33 strains isolated from samples from the Abéché market and 31 strains from the Bokoro, Mandélie and N'Djaména markets respectively. The sample numbers ranged from 1 to 28. Sample numbers 1–7 were from the Abéché market, 8–14 from the Bokoro market, 15–21 from the Mandélie market and 22–28 from the N'Djaména market. The strains were coded according to the food, the sample and the colony number (ka3–2: food: ka, sample: 3, number: 2). All isolates were Gram-positive, catalase-positive, caseinase-positive, spore-producing colonies (Fig. 1). Macroscopic examination on LB agar showed that 100% of the colonies were white in color with flat relief and the majority of which were irregular in outline and shape, variable in appearance and size. Microscopic observation showed that all isolates had rod shapes, the majority of which were motile, short in size, and with a mono-, diplo-, or chain-like clustering

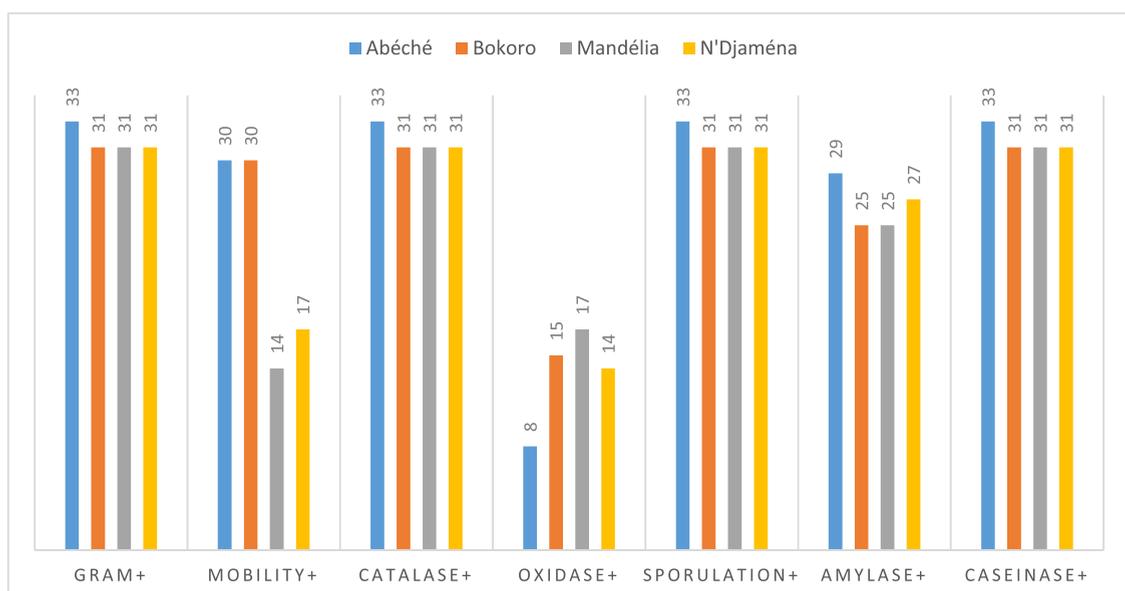


Fig. 1. Characteristics of isolates from Kawal.

Table 1
Antimicrobial and hemolytic activities of forty five strains.

| Market | Coded | Antibacterial activities | Antifungal activities | | | Hemolytic activities |
|-----------|--------|--------------------------|---------------------------|--------------------------------|------------------------------|----------------------|
| | | <i>M. luteus</i> LMG3293 | <i>Aspergillus flavus</i> | <i>Aspergillus parasiticus</i> | <i>Aspergillus fumigatus</i> | Blood gelose |
| Abéché | ka1-4 | 16.35±0.50 | 0 | 0 | 0 | + |
| Abéché | ka1-5 | 14.97±0.52 | 9.35±0.35 | 0 | 9.2±00 | - |
| Abéché | ka2-5 | 18.57±0.50 | 0 | 0 | 0 | - |
| Abéché | ka3-1 | 26.81±0.41 | 8.7±0.28 | 9.25±0.21 | 0 | + |
| Abéché | ka3-2 | 15.92±0.50 | 11.55±0.21 | 14.4±0.14 | 10.2±0.14 | + |
| Abéché | ka3-3 | 14.95±0.67 | 18.5±0.56 | 12.05±0.07 | 15.05±0.07 | + |
| Abéché | ka3-4 | 18.20±0.50 | 0 | 0 | 0 | - |
| Abéché | ka3-5 | 13.08±0.35 | 15.1±00 | 0 | 10.8±0.84 | - |
| Abéché | ka4-2 | 17.64±1.45 | 20.4±0.28 | 14.4±0.14 | 17.15±0.21 | - |
| Abéché | ka4-3 | 21.29±0.50 | 17.5±0.56 | 9.35±0.35 | 10.4±0.42 | - |
| Abéché | ka4-4 | 24.41±0.50 | 12.65±0.21 | 10±0.42 | 11.25±0.07 | + |
| Abéché | ka4-5 | 23.5±0.00 | 0 | 0 | 0 | + |
| Abéché | ka6-4 | 18.35±0.50 | 0 | 0 | 0 | + |
| Abéché | ka7-3 | 13.67±0.92 | 0 | 0 | 0 | + |
| Bokoro | ka9-1 | 18.45±0.15 | 0 | 0 | 0 | - |
| Bokoro | ka10-2 | 17.32±0.50 | 0 | 0 | 0 | - |
| Bokoro | ka10-3 | 15.90±0.50 | 0 | 0 | 0 | + |
| Bokoro | ka11-1 | 18.82±0.20 | 16.95±0.21 | 10.05±0.07 | 12.3±0.28 | - |
| Bokoro | ka12-2 | 19.90±0.50 | 11.15±0.35 | 0 | 9.2±0.14 | - |
| Bokoro | ka12-5 | 14.25±0.25 | 18.55±0.70 | 14.05±0.21 | 17.95±0.07 | + |
| Bokoro | ka13-3 | 16.97±0.15 | 0 | 0 | 0 | + |
| Mandélie | ka15-3 | 12.99±0.50 | 0 | 0 | 0 | - |
| Mandélie | ka15-5 | 22.80±0.50 | 16.95±1.62 | 13.1±0.28 | 15.95±0.49 | - |
| Mandélie | ka16-3 | 19.90±0.00 | 0 | 0 | 0 | - |
| Mandélie | ka17-2 | 17.00±0.19 | 14.1±0.98 | 13.5±0.28 | 9.45±0.21 | - |
| Mandélie | ka18-1 | 15.96±0.50 | 0 | 0 | 0 | - |
| Mandélie | ka18-2 | 18.11±0.50 | 0 | 0 | 0 | - |
| Mandélie | ka18-3 | 15.66±0.50 | 0 | 0 | 0 | - |
| Mandélie | ka19-2 | 12.28±0.41 | 0 | 0 | 0 | + |
| Mandélie | ka19-3 | 17.31±0.50 | 0 | 0 | 0 | - |
| Mandélie | ka21-4 | 24.21±0.11 | 0 | 0 | 0 | - |
| N'Djaména | ka22-3 | 11.37±0.12 | 0 | 0 | 0 | + |
| N'Djaména | ka23-2 | 27.55±0.50 | 0 | 10.1±00 | 0 | - |
| N'Djaména | ka23-3 | 16.75±0.25 | 0 | 0 | 0 | + |
| N'Djaména | ka23-5 | 11.60±0.25 | 0 | 0 | 0 | + |
| N'Djaména | ka24-2 | 17.84±0.50 | 0 | 0 | 0 | - |
| N'Djaména | ka24-3 | 16.69±0.11 | 0 | 0 | 0 | - |
| N'Djaména | ka26-1 | 25.35±0.22 | 0 | 0 | 0 | - |
| N'Djaména | ka26-4 | 17.16±0.10 | 0 | 0 | 0 | + |
| N'Djaména | ka26-5 | 14.11±0.48 | 0 | 0 | 0 | + |
| N'Djaména | ka27-2 | 13.33±0.55 | 0 | 0 | 0 | - |
| N'Djaména | ka27-3 | 14.16±0.53 | 0 | 0 | 0 | - |
| N'Djaména | ka28-3 | 27.98±0.37 | 0 | 0 | 0 | + |
| N'Djaména | ka28-4 | 24.72±0.50 | 0 | 0 | 0 | - |
| N'Djaména | ka28-5 | 21.43±0.28 | 0 | 0 | 0 | + |

Legend: Hemolytic activities: (-) no hemolysis, (+) presence of hemolysis.

pattern (Fig. 1). Thus, 57.14 % were motile, while 84.12 % produced amylase (Fig. 1). This indicates that the isolates belong to the genus *Bacillus*.

Antibacterial activities of strains

All 126 *Bacillus* sp strains isolated were tested for their ability to inhibit the growth of *M. luteus* LMG 3293. Forty five (45) strains were active against it (Table 1) and were retained for the next steps of the study. The strongest (27.98±0.37 mm) and weakest (11.37±0.12 mm) inhibition was observed in two strains (respectively ka28-3 and ka22-3) isolated from the market in N'Djaména.

Antifungal activities of strains

Among the 45 tested bacterial strains, 14 strains showed antifungal activity. Among them, 8 strains showed positive activity against the 3 mold strains tested, 4 strains were effective against 2 and 2 against one mold strain (Fig. 2 and Table 1). Supernatants of strains ka4-2 and ka12-5 strongly inhibited the growth of *Aspergillus flavus*. The diameters of the

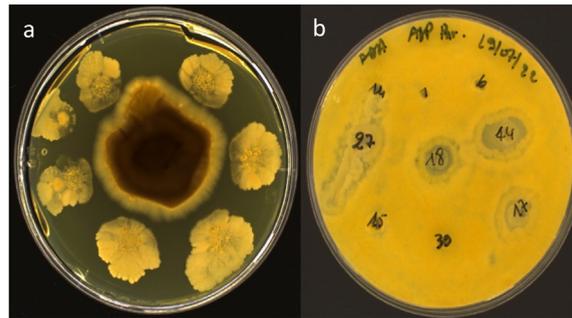


Fig. 2. Antifungal activity of *Bacillus* sp isolates 17; 18; 44: 17 against *Aspergillus parasiticus* (a) and *Aspergillus flavus* (b).

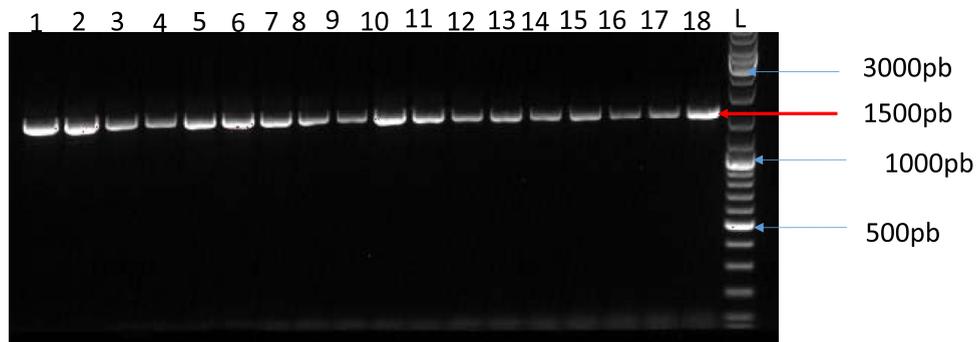


Fig. 3. 0.8% Agarose gel electrophoresis of the PCR products obtained after amplification of the gene encoding 16S rDNA in strains. L: ADN mix GeneRuler ladder (Thermo Scientific); 1: ka26-1; 2: ka15-3; 3: ka23-3; 4: ka19-3; 5: ka7-3; 6: ka13-3; 7: ka24-3; 8: ka12-2; 9: ka26-4; 10: ka4-5; L11: ka3-1; L12: ka23-2; L13: ka1-5; L14: ka12-5; L15: ka17-2; L16: ka10-2; L17: ka11-1; L18: ka4-3.

inhibition zones obtained with their supernatants were respectively 20.4 ± 0.28 mm and 18.55 ± 0.70 mm (Table 1). Strains Ka12-2, Ka3-5 and Ka1-5 inhibited the growth of *Aspergillus flavus* and *Aspergillus fumigatus*, while strain Ka3-1 inhibited the growth of *Aspergillus flavus* and *Aspergillus parasiticus* (Table 1). Strains Ka3-2, ka3-3, ka4-2, ka4-3, ka4-4, ka11-1, ka12-5, ka15-5 and Ka17-2 were all effective against *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus fumigatus* (Table 1).

Hemolytic activity of *Bacillus* spp

Bacillus sp strains with antimicrobial activity were selected to study the presence of hemolytic activity on blood agar medium after 40 h at 37 °C. Three types of hemolysis were observed, namely β , α and γ hemolysis. 19 out of 45 strains showed a zone of β and α hemolysis producing a clear halo around each colony indicating erythrocyte lysis (Table 1).

Identification

Profile of PCR products after migration

The Fig. 3 and 4 show the electrophoresis of the 16S gene PCR products, each isolate has a DNA band of about 1500 bp. S1 to S45 are the DNA bands of the different isolates.

Results of the BLASTn Bioinformatics Analysis of the Nucleotide Sequences of the 16S rRNA Genes of Isolates Having antimicrobial Activity

We used Blast results for the identification via NCBI / GenBank isolates of *Kawal* for the 16S rRNA genes (<https://www.ncbi.nlm.nih.gov/Genbank/update.html>). Different parameters of Blast such as Max score, Total Score, E. value and the percentage of similarity were used to identify the equivalent of the query strain in Gen Bank (Table 2). Forty-three bacteria of *Bacillus*-genus have been identified with a similarity between 98.12 and 99.86% against 16S rRNA genes of known species among the 45 isolated strains.

The sequences determined in the present study have been deposited under Genbank NCBI and will be available from the 31/01/2023 (SUB12243493). Their accession numbers are given in the last column of Table 2.

Distribution of isolates by species after sequencing and bio-informatics analysis

Sequencing of the genes coding for 16S rRNA allowed us to identify 43 *Bacillus* sp. isolates. Among them, we counted: 19 *Bacillus subtilis*, 5 *Bacillus licheniformis*, 5 *Bacillus tropicus*, 3 *Bacillus spizizenii*; 3 *Bacillus velezensis*, 2 *Bacillus amyloliqu-*

Table 2
Identification of *Bacillus* strains using 16S rRNA.

| Strain | Similarity (%) | Reference | Definition | Identified species | Accession number |
|--------|----------------|--------------|--|-----------------------------------|------------------|
| ka18-1 | 99.37% | NR_116,022.1 | <i>Bacillus amyloliquefaciens</i> strain BCRC 11,601 | <i>Bacillus amyloliquefaciens</i> | OP798045 |
| ka26-1 | 99.58% | NR_117,946.1 | <i>Bacillus amyloliquefaciens</i> strain MPA 1034 | <i>Bacillus amyloliquefaciens</i> | OP798023 |
| ka23-3 | 99.51% | NR_116,240.1 | <i>Bacillus velezensis</i> strain CBMB205 | <i>Bacillus velezensis</i> | OP798025 |
| ka19-3 | 99.51% | NR_116,240.1 | <i>Bacillus velezensis</i> strain CBMB205 | <i>Bacillus velezensis</i> | OP798026 |
| ka4-2 | 99.57% | NR_116,240.1 | <i>Bacillus velezensis</i> strain CBMB205 | <i>Bacillus velezensis</i> | OP798058 |
| ka4-5 | 99.37% | NR_112,686.1 | <i>Bacillus spizizenii</i> strain NBRC 101,239 | <i>Bacillus spizizenii</i> | OP798032 |
| ka1-4 | 99.37% | NR_112,686.1 | <i>Bacillus spizizenii</i> strain NBRC 101,239 | <i>Bacillus spizizenii</i> | OP798044 |
| ka4-3 | 99.23% | NR_112,686.1 | <i>Bacillus spizizenii</i> strain NBRC 101,239 | <i>Bacillus spizizenii</i> | OP798040 |
| ka12-2 | 99.93% | NR_118,996.1 | <i>Bacillus licheniformis</i> strain DSM 13 | <i>Bacillus licheniformis</i> | OP798030 |
| ka17-2 | 99.58% | NR_118,996.1 | <i>Bacillus licheniformis</i> strain DSM 13 | <i>Bacillus licheniformis</i> | OP798037 |
| ka10-3 | 99.22% | NR_118,996.1 | <i>Bacillus licheniformis</i> strain DSM 13 | <i>Bacillus licheniformis</i> | OP798042 |
| ka18-2 | 99.30% | NR_118,996.1 | <i>Bacillus licheniformis</i> strain DSM 13 | <i>Bacillus licheniformis</i> | OP798056 |
| ka21-4 | 99.30% | NR_118,996.1 | <i>Bacillus licheniformis</i> strain DSM 13 | <i>Bacillus licheniformis</i> | OP798047 |
| ka24-3 | 99.79% | NR_113,265.1 | <i>Bacillus subtilis</i> strain JCM 1465 | <i>Bacillus subtilis</i> | OP798029 |
| ka9-1 | 99.79% | NR_113,265.1 | <i>Bacillus subtilis</i> strain JCM 1465 | <i>Bacillus subtilis</i> | OP798041 |
| ka10-2 | 99.58% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798038 |
| ka1-5 | 99.72% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798035 |
| ka23-2 | 99.72% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798034 |
| ka11-1 | 99.51% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798039 |
| ka27-2 | 99.56% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798050 |
| ka15-5 | 99.56% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798051 |
| ka24-2 | 99.56% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798052 |
| ka19-2 | 99.86% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798053 |
| ka26-5 | 99.37% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798048 |
| ka3-5 | 99.58% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798043 |
| ka3-4 | 99.79% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798054 |
| ka28-3 | 99.64% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798060 |
| ka28-4 | 99.64% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798061 |
| ka27-3 | 99.65% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798062 |
| ka2-5 | 99.43% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798063 |
| ka3-3 | 99.57% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798064 |
| ka28-5 | 99.72% | NR_027552.1 | <i>Bacillus subtilis</i> strain DSM 10 | <i>Bacillus subtilis</i> | OP798065 |
| ka15-3 | 99.30% | NR_157,736.1 | <i>Bacillus tropicus</i> strain MCCC | <i>Bacillus tropicus</i> | OP798024 |
| ka3-2 | 99.65% | NR_157,736.1 | <i>Bacillus tropicus</i> strain MCCC 1A01406 | <i>Bacillus tropicus</i> | OP798055 |
| ka22-3 | 99.44% | NR_157,736.1 | <i>Bacillus tropicus</i> strain MCCC 1A01406 | <i>Bacillus tropicus</i> | OP798057 |
| ka6-4 | 99.36% | NR_157,736.1 | <i>Bacillus tropicus</i> strain MCCC 1A01406 | <i>Bacillus tropicus</i> | OP798049 |
| ka3-1 | 99.30% | NR_157,736.1 | <i>Bacillus tropicus</i> strain MCCC 1A01406 | <i>Bacillus tropicus</i> | OP798033 |
| ka12-5 | 99.58% | NR_115,526.1 | <i>Bacillus cereus</i> strain IAM 12,605 | <i>Bacillus cereus</i> | OP798036 |
| ka23-5 | 99.37% | NR_152,692.1 | <i>Bacillus wiedmannii</i> strain FSL W8-0169 | <i>Bacillus wiedmannii</i> | OP798059 |
| ka7-3 | 98.12% | NR_157,609.1 | <i>Bacillus haynesii</i> strain NRRL B-41,327 | <i>Bacillus haynesii</i> | OP798027 |
| ka13-3 | 98.12% | NR_157,609.1 | <i>Bacillus haynesii</i> strain NRRL B-41,327 | <i>Bacillus haynesii</i> | OP798028 |
| ka26-4 | 99.79% | NR_113,945.1 | <i>Bacillus safensis</i> strain NBRC 100,820 | <i>Bacillus safensis</i> | OP798031 |
| ka4-4 | 99.44% | NR_104,919.1 | <i>Bacillus tequilensis</i> strain 10b | <i>Bacillus tequilensis</i> | OP798046 |

uofaciens, 2 *Bacillus haynesii*, 1 *Bacillus cereus*, 1 *Bacillus safensis*, 1 *Bacillus tequilensis* and 1 *Bacillus wiedmannii* (Fig. 4). *Bacillus subtilis* was the most predominant specie (44% of isolates).

Phylogenetics of studied strains

The Fig. 5 shows the phylogenetic tree constructed from the nucleotide sequences of the 16S rRNA encoding genes of the *Bacillus* strains isolated from *kawal*. Alignment of all sequences (query sequences and homologs) was performed by clustalW (<http://www.ebi.ac.uk/clustalw>) [24]. The phylogenetic tree was produced using MEGA 11 software [22]. We used *Escherichia coli* strain U 5/41 (NR_024570.1) as an out-group. Phylogenetic tree analysis revealed that strains *B. subtilis* (ka10-2, ka3-4, ka9-1, ka23-4, ka11-1, ka1-5, ka26-5, ka23-2), *B. spizizenii* (ka4-5, ka4-3, ka1-4), *B. tequilensis* (ka4-4), *B. velezensis* (ka23-3, ka19-3), *B. amyloliquefaciens* (ka26-1, ka18-1); *B. haynesii* (ka7-3, ka13-3), and *B. licheniformis* (ka17-2, ka12-2, ka10-3, ka21-4) are closely related to *Bacillus subtilis* (Fig. 5). Similarly, group composed of *B. subtilis* (ka3-4, ka19-2, ka27-2, ka15-5, ka24-2, ka28-5, ka28-3, ka2-5, ka28-4, ka27-3, ka3-3), *B. velezensis* ka4-2, *B. wiedmannii* ka23-5, *B. licheniformis* ka18-2, *B. safensis* ka26-4 and *B. tropicus* (ka22-3, ka3-2) can be related to any *Bacillus* sp. member of “*B. subtilis* clade” or “*B. cereus* clade”. However, group composed of *B. tropicus* (ka15-3, ka3-1, ka6-4) and *B. cereus* ka12-5 have a very close phylogenetic relationship to *Bacillus cereus* member (Fig. 5).

Lipopeptide detected by MALDI-ToF-MS

MALDI-ToF-MS analysis of whole cells of the *Bacillus* strains that showed antimicrobial activities allowed the identification of the lipopeptides responsible for these activities. Three types of lipopeptide compounds with different masses (m/z)

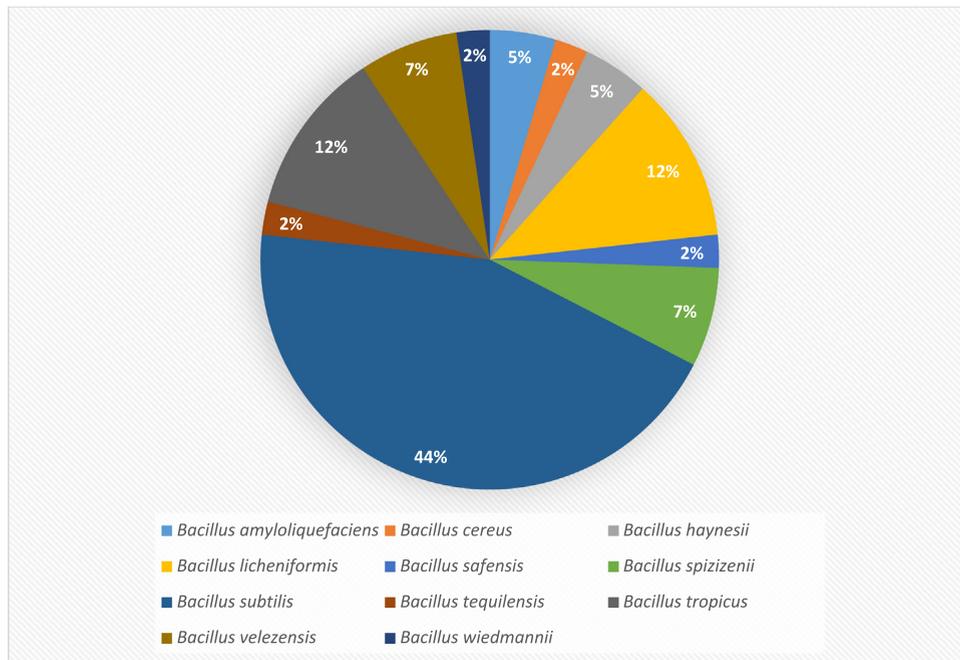


Fig. 4. Proportion of isolated *Bacillus* species.

were detected in these strains: 1000–1200 for surfactins and iturins, 1400–1600 for fengycin and 600–1000 for kurstakins [25]. Among the 43 *Bacillus* strains identified, 37 were found to be able to produce antimicrobial lipopeptides. Only one strain produced kurstakin (*B. cereus* ka12–5), 5 strains produced iturin, 10 strains produced fengycin, 36 produced surfactin and 2 other types of lipopeptides (strains ka12–5 and ka3–2) (Table 3). Among the iturin-producing strains, mass peaks close to mycosubtilin were detected in *B. velezensis* ka4–2, while others close to mojavensin were detected in *B. subtilis* ka3–3 and *B. wiedmannii* ka23–5. Mass peaks corresponding to fengycin A and B were detected in *B. subtilis* ka23–2 and *B. velezensis* ka4–2 respectively. Furthermore, the study showed that 9 strains produced two families of lipopeptides. Fengycin and surfactin were detected in *B. licheniformis* ka12–2, *B. subtilis* ka23–2, *B. subtilis* ka1–5, *B. spizizenii* ka4–3, *B. subtilis* ka3–5, *B. tequilensis* ka4–4, *B. subtilis* ka15–5 and *B. subtilis* ka28–3. The *B. subtilis* ka3–3 strain produced mojavensin and surfactin at the same time. Only *B. cereus* ka12–5, *B. subtilis* ka11–1 and *B. velezensis* ka4–2 strains showed the ability to secrete three lipopeptides at the same time, kurstakin/iturin/surfactin, iturin/surfactin/fengycin and mycosubtilin/surfactin/fengycin respectively (Table 3).

Discussion

Microbiological analysis of the *kawal*, shows an important spore microflora mainly composed of *Bacillus*. Most representative species were *B. subtilis* (44%), *B. licheniformis* (12%) and *B. tropicus* (12%). Semi-aerobic conditions prevailing during the fermentation process favor the predominance of facultative anaerobic germs such as *B. licheniformis*, *B. tropicus* and *B. velezensis* [26]. Fermentation of *Senna obtusifolia* leaves is an exothermic process [1]. It was shown that during the 3-day fermentation of *Ugba*, an African fermented bean food, the increase in temperature (26°C to 46°C) corresponds to an increase in microbial population and pH (7.04 to 8.55) [27]. pH rise during fermentation would be linked to the action of microbial proteases [28]. *B. amyloliquefaciens*; *B. cereus*; *B. haynesii*; *B. licheniformis*; *B. safensis*; *B. spizizenii*; *B. subtilis*; *B. tequilensis*; *B. tropicus*; *B. velezensis* and *B. wiedmannii* found in *kawal* have such enzymes and are able to degrade the proteins of *Senna obtusifolia* leaves [28]. This proteolytic activity leads to a release of peptides, ammonia and contributes positively to the flavor and aroma of the fermented product [29]. Implications of *B. subtilis*, *B. amyloliquefaciens*, *B. velezensis*, *B. licheniformis*, *B. tropicus*, *B. cereus*, *B. spizizenii* and *B. safensis* are reported by different authors in the fermentation of *soumbala* and *bikalga* [26]. However, *B. haynesii* and *B. tequilensis* which are commonly found in soil samples have not yet been reported. Their presence in the *kawal* could be explained by contamination.

Our work has focused on the detection of secondary metabolites, in particular lipopeptides that could be involved in the biopreservation and functional properties of *kawal*. Two techniques were used on the forty-three (43) *Bacillus* strains selected on the basis of their antimicrobial activities. All strains were able to inhibit the growth of *Micrococcus luteus* and some fungal strains (*Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus fumigatus*). In addition, surfactin (84%), fengycin (23%) and iturin (13%) were detected in our strains. However, kurstakin was only detected in one strain, *B. cereus* ka12–5.

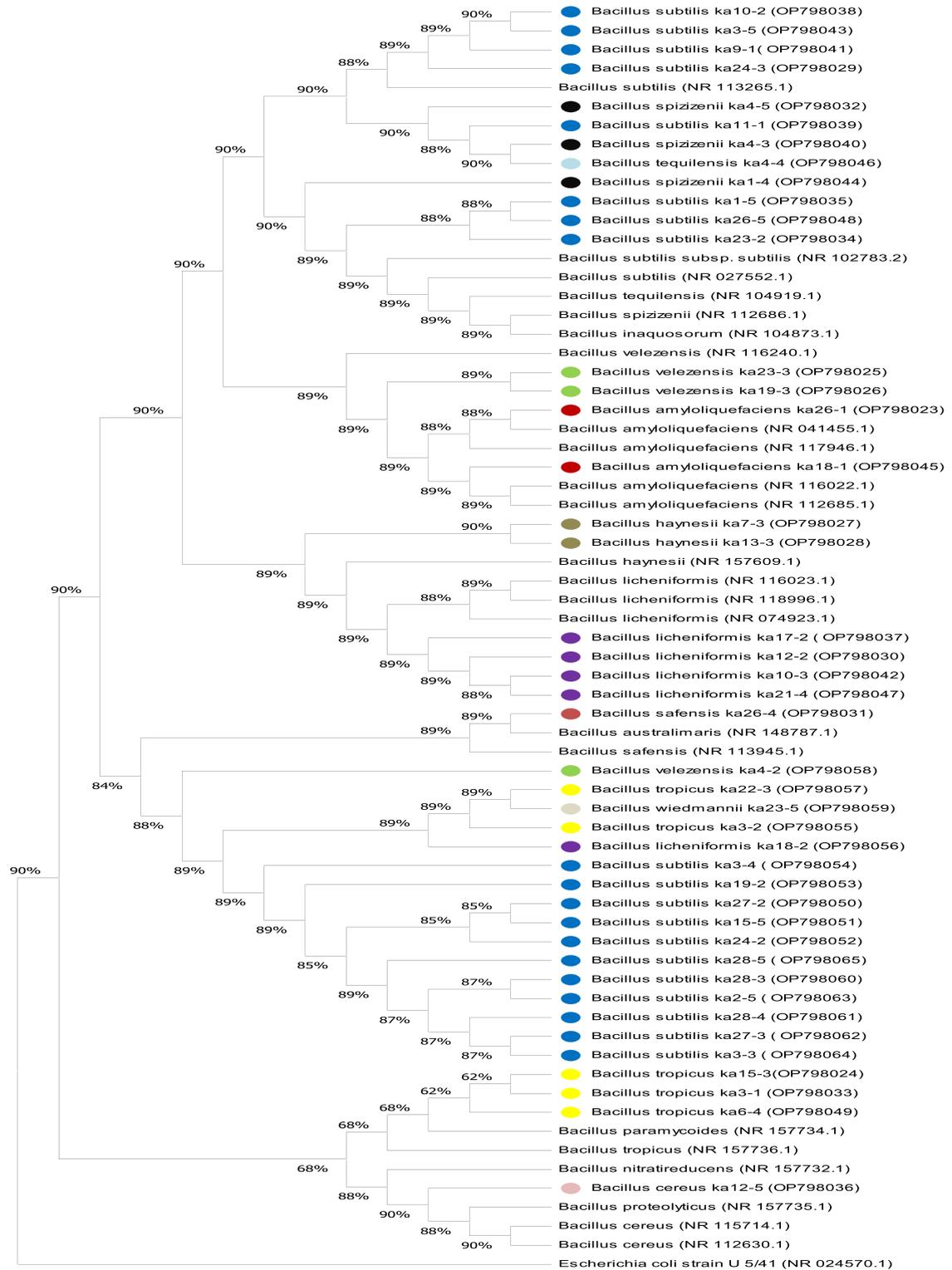


Fig. 5. Phylogenetic tree constructed from the alignment of nucleotide sequences genes coding for the 16S rRNA of 43 *Bacillus* strains isolated from *kawal*. The phylogenetic tree was constructed using MEGA 11 by the Neighborjoining method and *Escherichia coli* strain U 5/41 (NR_024570.1) was used as an outgroup.

Table 3
Lipopeptides produced by the antimicrobial *Bacillus* strain detected by MALDI-ToF-MS analysis.

| Strain | Species | Peak mass (m/z) | Antibiotics | Production intensity |
|--------|-----------------------------------|---|------------------------|----------------------|
| ka18-1 | <i>Bacillus amyloliquefaciens</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka26-1 | <i>Bacillus amyloliquefaciens</i> | 1058.6, 1074.6 [Na+, K+] surfactin C15 or pumilacidin C14, C15 | surfactin /pumilacidin | ++ |
| ka19-3 | <i>Bacillus velezensis</i> | 1057.4 [Na+] surfactin C15 or lichenysin C15 | surfactin/lichenysin | +++ |
| ka4-2 | <i>Bacillus velezensis</i> | 1008.6, 1058.6 [H+, Na+] surfactin C15, C13 or pumilacidin C15 | surfactin/pumilacidin | +++ |
| ka4-2 | <i>Bacillus velezensis</i> | 1107, 1123 [Na+, K+] mycosubtilin C17, iturin A C17, bacillomycin F C16 | iturin | ++ |
| ka4-2 | <i>Bacillus velezensis</i> | 1475.8, 1477 [H+] fengycin B C15 | Fengycin B | ++ |
| ka4-5 | <i>Bacillus spizizenii</i> | 1058.6, 1074.6 [Na+, K+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | ++ |
| ka1-4 | <i>Bacillus spizizenii</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka4-3 | <i>Bacillus spizizenii</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka4-3 | <i>Bacillus spizizenii</i> | 1513.7, 1527.8, 1529.8 [Na+, K+] fengycin A C16, C17, C18 or Fengycin B C15, C16, C17, C18 | fengycin A or B | + |
| ka12-2 | <i>Bacillus licheniformis</i> | 1058.6, 1074.6, 1100.6, 1116.6 [Na+, K+] surfactin C15 or pumilacidin C14, C17 | surfactin/pumilacidin | +++ |
| ka12-2 | <i>Bacillus licheniformis</i> | 1513.8, 1515.8, 1527.8, 1529.8, 1543.8 [Na+, K+] fengycin A C18 or fengycin B C16, C17, C18 | fengycin A or B | + |
| ka10-3 | <i>Bacillus licheniformis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | ++ |
| ka18-2 | <i>Bacillus licheniformis</i> | 1058.6, 1074.6 [Na+, K+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | ++ |
| ka21-4 | <i>Bacillus licheniformis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka24-3 | <i>Bacillus subtilis</i> | 1058.6, 1074.6 [Na+, K+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka9-1 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka10-2 | <i>Bacillus subtilis</i> | 1044.6, 1058.6, 1060.6, 1074.6 [Na+, K+] surfactin C14, C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka1-5 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka1-5 | <i>Bacillus subtilis</i> | 1457.7, 1455.7, 1497.7, 1499.8 [Na+, K+] fengycin A C14 or fengycin B C15, C16, C17 | fengycin A or B | + |
| ka23-2 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka23-2 | <i>Bacillus subtilis</i> | 1513.6, 1527.8, 1529 [Na+, K+] fengycin A C18 | fengycin A | + |
| ka11-1 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka11-1 | <i>Bacillus subtilis</i> | 1106, 1122 [Na+, K+] iturin C1 (C17) | iturin C | +++ |
| ka11-1 | <i>Bacillus subtilis</i> | 1513.8, 1515.8, 1527.8, 1529.8, 1543.8 [Na+, K+] fengycin A C17, C18 or fengycin B C16, C17 | fengycin A or B | + |
| ka27-2 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | + |
| ka15-5 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka15-5 | <i>Bacillus subtilis</i> | 1513.8, 1515.8, 1527.8, 1529.8, 1543.8 [Na+, K+] fengycin A C17, C18 or fengycin B C16, C17 | fengycin A or B | +++ |
| ka19-2 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | ++ |
| ka3-5 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka3-5 | <i>Bacillus subtilis</i> | 1513.8, 1515.8, 1527.8, 1529.8, 1543.8 [Na+, K+] fengycin A C17, C18 or fengycin B C16, C17 | fengycin A or B | ++ |
| ka3-4 | <i>Bacillus subtilis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | ++ |
| ka28-3 | <i>Bacillus subtilis</i> | 1058.6, 1074.6, 1100.6, 1116.6 [H+, Na+, K+] surfactin C15 or pumilacidin C14, C15, C17 | surfactin/pumilacidin | +++ |
| ka28-3 | <i>Bacillus subtilis</i> | 1513.8, 1515.8, 1527.8, 1529.8, 1543.8 [Na+, K+] fengycin A C17, C18 or fengycin B C16, C17 | fengycin A or B | + |
| ka28-4 | <i>Bacillus subtilis</i> | 1058.6, 1074.6, 1100.6, 1116.6 [H+, Na+, K+] surfactin C15 or pumilacidin C14, C15, C17 | surfactin/pumilacidin | +++ |
| ka27-3 | <i>Bacillus subtilis</i> | 1058.6, 1074.6, 1100.6, 1116.6 [H+, Na+, K+] surfactin C15 or pumilacidin C14, C15, C17 | surfactin/pumilacidin | +++ |

(continued on next page)

Table 3 (continued)

| Strain | Species | Peak mass (m/z) | Antibiotics | Production intensity |
|--------|-----------------------------|---|-----------------------|----------------------|
| ka2-5 | <i>Bacillus subtilis</i> | 1058.6, 1074.6, 1100.6, 1116.6 [H+, Na+, K+] surfactin C15 or pumilacidin C14, C15, C17 | surfactin/pumilacidin | +++ |
| ka3-3 | <i>Bacillus subtilis</i> | 1030.6, 1046.6 [Na+, K+] surfactin C13, C14 or C15 | surfactin | +++ |
| ka3-3 | <i>Bacillus subtilis</i> | 1084.5, 1106.5 [H+, Na+] mojavensin | mojavensin | + |
| ka28-5 | <i>Bacillus subtilis</i> | 1058.6, 1074.6, 1100.6, 1116.6 [H+, Na+, K+] surfactin C15 or pumilacidin C14, C15, C17 | surfactin/pumilacidin | +++ |
| ka3-2 | <i>Bacillus tropicus</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | + |
| ka3-2 | <i>Bacillus tropicus</i> | 2210.9, 2227.9, 2239.9, 3354.9, 2054.7 [Na+] | other | +++ |
| ka6-4 | <i>Bacillus tropicus</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka3-1 | <i>Bacillus tropicus</i> | 1035, 1058 [H+, Na+], surfactin C15 or pumilacidin C14 | surfactin/pumilacidin | +++ |
| ka12-5 | <i>Bacillus cereus</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | + |
| ka12-5 | <i>Bacillus cereus</i> | 1106, 1120.5, 1122.5, 1136.5 [Na+, K+] iturin C | iturin C | + |
| ka12-5 | <i>Bacillus cereus</i> | 874, 940, 956, 1011 [Na+, K+] kurstakin | kurstakin | +++ |
| ka12-5 | <i>Bacillus cereus</i> | 2484.8 [Na+] | other | +++ |
| ka23-5 | <i>Bacillus wiedmannii</i> | 1084.5, 1106.5 [H+, Na+] mojavensin | mojavensin | +++ |
| ka7-3 | <i>Bacillus haynesii</i> | 1057.4 [Na+] surfactin C15 or lichenysin C15 | surfactin | +++ |
| ka13-3 | <i>Bacillus haynesii</i> | 1057.4 [Na+] surfactin C15 or lichenysin C15 | surfactin | +++ |
| ka26-4 | <i>Bacillus safensis</i> | 1058.6, 1074.6, 1100.6, 1116.6 [H+, Na+, K+] surfactin C15 or pumilacidin C14, C15, C17 | surfactin/pumilacidin | ++ |
| ka4-4 | <i>Bacillus tequilensis</i> | 1036.6, 1058.1 [H+, Na+] surfactin C15 or pumilacidin C14, C15 | surfactin/pumilacidin | +++ |
| ka4-4 | <i>Bacillus tequilensis</i> | 1513.8, 1515.8, 1527.8, 1529.8, 1543.8 [Na+, K+] fengycin A C17, C18 or fengycin B C16, C17 | fengycin A or B | + |

Legend: (+) low production, (++) medium production, (+++) high production

Many *Bacillus* species are known to produce a wide diversity of antimicrobial peptides [30]. These antimicrobial peptides are most widely used as food additives for preservation [3]. They are involved in both spontaneous fermentation and large scale fermentation processes for the preservation and transformation of many raw materials from animals and vegetables [3]. Our results support the findings in which iturin, surfactin and fengycin are among the most common lipopeptide antibiotics found in a wide variety of *Bacillus sp.* including *B. subtilis* as the model strain [31]. Additionally, kurstakin antibiotics have been associated with the inhibition of pathogenic microbial through the destruction of the structure and permeability of cell membrane [32]. In our study, surfactin, iturin, fengycin, mojavensin and kurstakin have been implicated in the microbial activity against *Micrococcus luteus*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus fumigatus*. Kaboré et al. [33] had reported the antagonistic activity of *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus circulans* and *Bacillus megaterium* strains isolated from *Maari* against strains of *Aspergillus flavus*, *Aspergillus ochraceus* and *Penicillium roqueforti*.

According to Labiadh et al. [31], iturin C, bacillomycin D, fengycin and Kurstakin are involved in the antibacterial activity of *Bacillus sp.* Quantitative analysis of lipopeptide production by the isolated strains showed that 55% produced surfactins at high concentrations. These concentrations are sufficient to contribute to the preservation of these fermented condiments and their functional properties [17]. Indeed, Ouoba et al. [29] concluded that certain strains of *B. cereus* could produce toxins during the *soumbala* and *bikalga* fermentation whereas Arslan et al. [34] reported that *B. cereus* toxin production can be affected by other microorganism and fermentation. Surfactins are bioactive molecules that inhibit the growth of toxin-producing strains [17]. In addition, lipopeptide could play other roles in the food products [35]. Indeed, its remarkable emulsifying and biosurfactant properties could, for example, impact the organoleptic properties of the products by modifying, even at low concentrations, the solubility of different hydrophobic compounds [17]. Microbial surfactants are of particular interest because they are biodegradable and can be produced through fermentation on renewable substrates [36]. The results of this study demonstrated the high diversity of lipopeptides among *Bacillus* strains of *kawal* and their antimicrobial capacity. Therefore, it is a food that could play an essential role in the health of the consumer by fighting effectively against diseases with regard to antibiotics and antifungals that it contains.

Conclusion

Our results highlight the potential production of lipopeptides by different strains of *Bacillus* obtained from *kawal* and highlight their antimicrobial potential. Additionally, the results of this study emphasize the need of new research focused on biosurfactant lipopeptides, which can be used as bioconservation of food.

Declaration of competing interest

The authors declare that there is no conflict of interest and confirm that this manuscript does not infringe any other copyright or property rights. All authors agreed to publication of the work.

Acknowledgements

This work was supported by SCAC (Service de Cooperation et d'Action Culturelle). We express our gratitude to all the authors who contributed to the achievement of the objectives of this study. We also thank the Laboratory UMRt1158 BioEcoAgro of University Lille, north of France and the Cooperation Service of the French Embassy in Burkina Faso for their support.

References

- [1] I.L. Abakar, F. Guira, F. Tapsoba, C. Zongo, H. Abdoullahi, A. Tidjani, Le *kawal*, un condiment à base de feuilles fermentées de *Senna obtusifolia*, Afr. J. Food Agric. Nutr. 19 (2) (2019) 14244–14260, doi:[10.18697/ajfand.85.17435](https://doi.org/10.18697/ajfand.85.17435).
- [2] FAO. Note d'orientation Développer la chaîne agroalimentaire en Messages clés. 2012: 1–4.
- [3] H. Cissé, B. Kagambèga, A. Sawadogo, A. Tankoano, G. Sangaré, Y. Traoré, I.L. Ouoba, Molecular characterization of *Bacillus*, lactic acid bacteria and yeast as potential probiotic isolated from fermented food, Sci. Afr. 6 (2019) e00175, doi:[10.1016/j.sciaf.2019.e00175](https://doi.org/10.1016/j.sciaf.2019.e00175).
- [4] Y. Dabiré, N.S. Somda, K. Marius, C.B. Compaoré, I. Mogmenga, L.I. Ezeogu, S. Alfred, J.O. Ugwuanyi, Assessment of probiotic and technological properties of *Bacillus* spp. isolated from Burkinaabe Soumbala, BMC Microbiol. 22 (1) (2022) 1–13, doi:[10.1186/s12866-022-02642-7](https://doi.org/10.1186/s12866-022-02642-7).
- [5] E. Taale, A. Hissein, A. Tankoano, Traditional technologies and probiotic properties of *Bacillus* strains isolated from *kawal* -a Chad traditional fermented food condiment, J. Food Technol. Res. 6 (2) (2019) 57–71, doi:[10.18488/journal.58.2019.62.57.71](https://doi.org/10.18488/journal.58.2019.62.57.71).
- [6] S. Caulier, C. Nannan, A. Gillis, F. Licciardi, C. Bragard, Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group, Front. Microbiol. 10 (2019) 1–19, doi:[10.3389/fmicb.2019.00302](https://doi.org/10.3389/fmicb.2019.00302).
- [7] F. Coutte, D. Lecouturier, K. Dimitrov, J.S. Guez, F. Delvigne, P. Dhulster, Microbial lipopeptide production and purification bioprocesses, current progress and future challenges, Biotechnol. J. 12 (7) (2017) 1–10, doi:[10.1002/biot.201600566](https://doi.org/10.1002/biot.201600566).
- [8] K. Jung-ae, S. Jeong-sup, K. Pyoung, K. Dae-hyuk, *Bacillus velezensis* TSA32-1 as a promising agent for biocontrol of plant pathogenic fungi, J. Fungi 8 (10) (2022) 1–15, doi:[10.3390/jof8101053](https://doi.org/10.3390/jof8101053).
- [9] C. Luo, X. Liu, H. Zhou, X. Wang, Nonribosomal peptide synthase gene clusters for lipopeptide biosynthesis in *Bacillus subtilis* 916 and their phenotypic functions, Appl. Environ. Microbiol. 81 (1) (2015) 422–431, doi:[10.1128/AEM.02921-14](https://doi.org/10.1128/AEM.02921-14).
- [10] J. Davel, S. Lemièrre, F. Coutte, F. Krier, N.V. Hese, M. Béchet, N. Sourdeau, M. Höfte, A. Leprêtre, Mycosubtilin and surfactin are efficient, low ecotoxicity molecules for the biocontrol of lettuce downy mildew, Appl. Microbiol. Biotechnol. 98 (14) (2014) 6255–6264, doi:[10.1007/s00253-014-5663-1](https://doi.org/10.1007/s00253-014-5663-1).
- [11] M. Ongena, P. Jacques, *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol, Trends Microbiol. 16 (3) (2007) 115–125, doi:[10.1016/j.tim.2007.12.009](https://doi.org/10.1016/j.tim.2007.12.009).
- [12] F. Jacques. Biosurfactants: A General Overview. 2011: 1–211.
- [13] F. Peypoux, F. Besson, G. Michel, L. Delcambre, B.C. Das, Structure de l'iturine C de *Bacillus subtilis*, Tetrahedron 34 (8) (1978) 1147–1152, doi:[10.1016/0040-4020\(78\)80138-0](https://doi.org/10.1016/0040-4020(78)80138-0).
- [14] M. Béchet, T. Caradec, W. Hussein, A. Abderrahmani, M. Chollet, V. Leclère, T. Dubois, D. Lereclus, M. Pupin, Structure, biosynthesis, and properties of kurstakins, nonribosomal lipopeptides from *Bacillus* spp, Appl. Microbiol. Biotechnol. 907 (2012) 593–600, doi:[10.1007/s00253-012-4181-2](https://doi.org/10.1007/s00253-012-4181-2).
- [15] Y. Huan, Q. Kong, H. Mou, H. Yi, Antimicrobial peptides: classification, design, application and research progress in multiple, Front. Microbiol. 11 (2020) 1–21, doi:[10.3389/fmicb.2020.582779](https://doi.org/10.3389/fmicb.2020.582779).
- [16] S. Olishchevska, A. Nickzad, E. Déziel, *Bacillus* and *Paenibacillus* secreted polyketides and peptides involved in controlling human and plant pathogens, Appl. Microbiol. Biotechnol. 103 (3) (2019) 1189–1215, doi:[10.1007/s00253-018-9541-0](https://doi.org/10.1007/s00253-018-9541-0).
- [17] A. Savadogo, A. Tapi, M. Chollet, B. Wathelet, A.S. Traoré, Identification of surfactin producing strains in Soumbala and Bikalga fermented condiments using Polymerase Chain Reaction and Matrix Assisted Laser Desorption /Ionization-Mass Spec, Int. J. Food Microbiol. 151 (3) (2017) 299–306, doi:[10.1016/j.ijfoodmicro.2011.09.022](https://doi.org/10.1016/j.ijfoodmicro.2011.09.022).
- [18] J.R. Tagg, A.S. Dajani. Bacteriocins of Gram-Positive Bacteria. 1976; 40(3): 722–756.
- [19] H. Cisse, A. Savadogo, E. Taale, F. Tapsoba, F. Guira, C. Zongo, Y. Traore, Influence des substrats carbonés et minéraux sur l'activité des substances BLIS (Bacteriocin-Like Inhibitory Substances) produites par des souches de *Bacillus* isolées à partir d'aliments fermentés au Burkina Faso, J. Appl. Biosci. 106 (2016) 10236–10248.
- [20] A. Tapi, M. Chollet-imberty, B. Scherens, B. Scherens, P. Jacques, New approach for the detection of non-ribosomal peptide synthetase genes in *Bacillus* strains by polymerase chain reaction, Appl. Microbiol. Biotechnol. 85 (5) (2010) 1521–1531, doi:[10.1007/s00253-009-2176-4](https://doi.org/10.1007/s00253-009-2176-4).
- [21] H. Compaore, S. Samandoulougou, L.Y. Ware, A. Bambara, H. Ratongue, I. Sawadogo, Identification of *Aspergillus* section Flavi and Fumigati in maize grown in Burkina Faso, Int. J. Biosci. 18 (2021) 25–36, doi:[10.12692/ijb/18.6.25-36](https://doi.org/10.12692/ijb/18.6.25-36).
- [22] K. Tamura, G. Stecher, MEGA11 : molecular evolutionary genetics analysis version 11, Mol. Biol. Evolution 38 (7) (2021) 3022–3027, doi:[10.1093/molbev/msab120](https://doi.org/10.1093/molbev/msab120).
- [23] J. Vater, B. Kablitz, C. Wilde, P. Franke, N. Mehta, S.S. Cameotra, Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge, Appl. Environ. Microbiol. 68 (12) (2002) 6210–6219.
- [24] J.D. Thompson, T.J. Gibson, Multiple sequence alignment using ClustalW and ClustalX, Curr. Protoc. Bioinforma. 00 (1) (2003) 1–22, doi:[10.1002/0471250953.bi0203s00](https://doi.org/10.1002/0471250953.bi0203s00).
- [25] M. Fanaei, J. Jurcic, Kristina, Detection of simultaneous production of kurstakin, fengycin and surfactin lipopeptides in *Bacillus mojavensis* using a novel gel – based method and MALDI – TOF spectrometry, World J. Microbiol. Biotechnol. 37 (6) (2021) 1–11, doi:[10.1007/s11274-021-03064-9](https://doi.org/10.1007/s11274-021-03064-9).
- [26] Y. Dabiré, N.S. Somda, M.K. Somda, I. Mogmenga, A.K. Traoré, L.I. Ezeogu, A.S. Traoré, J.O. Ugwuanyi. Molecular identification and safety assessment of *Bacillus* strains isolated from Burkinaabe traditional condiment ' Soumbala.' Ann. Microbiol. 2022; 72(10): doi:[10.1186/s13213-022-01664-w](https://doi.org/10.1186/s13213-022-01664-w).
- [27] A.I. Lawane, S. Aly, T. François, G. Flibert, T. Yves, Identification and biochemical analysis of microorganisms involved in the fermentation of *Kawal*-A traditional fermented sicklepod leaves (*Senna obtusifolia*), Int. J. Curr. Microbiol. App. Sci. 5 (9) (2016) 261–270.
- [28] I. Ahaotu, A. Anyogu, O.H. Njoku, N.N. Odu, J.P. Sutherland, Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla Benth*) for production of Ugba, Int. J. Food Microbiol. 162 (1) (2013) 95–104, doi:[10.1016/j.ijfoodmicro.2013.01.001](https://doi.org/10.1016/j.ijfoodmicro.2013.01.001).
- [29] L.I. Ouoba, B. Diawara, L. Jespersen, Antimicrobial activity of *Bacillus subtilis* and *Bacillus pumilus* during the fermentation of African locust bean (*Parkia biglobosa*) for Soumbala production, J. Appl. Microbiol. 102 (4) (2006) 963–970, doi:[10.1111/j.1365-2672.2006.03156.x](https://doi.org/10.1111/j.1365-2672.2006.03156.x).
- [30] A.K. Saxena, M. Kumar, H. Chakdar, N. Anuroopa, *Bacillus* species in soil as a natural resource for plant health and nutrition, J. Appl. Microbiol. 128 (6) (2020) 1583–1594, doi:[10.1111/jam.14506](https://doi.org/10.1111/jam.14506).

- [31] M. Labiadh, S. Dhaouadi, M. Chollet, G. Chataigne, C. Tricot, P. Jacques, S. Flahaut. Rhizosphere Antifungal lipopeptides from *Bacillus* strains isolated from rhizosphere of Citrus trees. 2021; 19: 100399. doi:[10.1016/j.rhisph.2021.100399](https://doi.org/10.1016/j.rhisph.2021.100399).
- [32] A. Abderrahmani, A. Tapi, F. Nateche, M. Chollet, V. Leclère, B. Wathelet, H. Hocine, Bioinformatics and molecular approaches to detect NRPS genes involved in the biosynthesis of kurstakin from *Bacillus thuringiensis*, Appl. Microbiol. Biotechnol. 92 (3) (2011) 571–581, doi:[10.1007/s00253-011-3453-6](https://doi.org/10.1007/s00253-011-3453-6).
- [33] D. Kaboré, M. Gagnon, D. Roy, H. Sawadogo-Lingania, B. Diawaraa, Rapid screening of starter cultures for Maari based on antifungal properties, Microbiol. Res. 207 (2018) 66–74, doi:[10.1016/j.micres.2017.11.005](https://doi.org/10.1016/j.micres.2017.11.005).
- [34] F. Ozdemir, S. Arslan, Molecular characterization and toxin profiles of *Bacillus spp* . Isolated from retail fish and ground beef, J. Food Sci. 84 (3) (2019), doi:[10.1111/1750-3841.14445](https://doi.org/10.1111/1750-3841.14445).
- [35] K. Salek, S.R. Euston, Sustainable microbial biosurfactants and bioemulsifiers for commercial exploitation, Process Biochem. 85 (2019) 143–155, doi:[10.1016/j.procbio.2019.06.027](https://doi.org/10.1016/j.procbio.2019.06.027).
- [36] S. Rath, R.K. Srivastava, Biosurfactants production and their commercial importance, Environ. Agric. Microbiol. Adv. Appl. (2021) 197–218.